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Research Article

Proinflammatory effects of copper deficiency on neutrophils and lung endothelial cells

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Summary Dietary copper deficiency increases the accumulation of circulating neutrophils in the rat lung microcirculation. This process includes neutrophil adhesion to, migration along, and emigration though the vascular endothelium. The current study was designed to examine the role of copper in each of these steps. Neutrophils were isolated from rats fed either a copper-adequate (CuA, 6.1 μg Cu/g diet) or copper-deficient diet (CuD, 0.3 μg Cu/g diet) for 4 weeks. First, transient and firm adhesion of neutrophils to P-selectin in a flow chamber showed there were more adhered CuD neutrophils than CuA ones. This effect is probably caused by the increased expression of CD11b that was observed in the current study. Second, the evaluation of neutrophil migration under agarose showed that the CuD neutrophils moved farther than the CuA group in response to IL-8 but not *f*MLP; this suggests an increased sensitivity to a CD11/CD18-independent signalling pathway. Third, the contractile mechanism of endothelial cells was studied. Elevated F-actin formation in Cu-chelated lung microvascular endothelial cells suggests that neutrophil emigration may be promoted by enhanced cytoskeletal reorganization of the endothelium during copper deficiency. Combined, these results support the theory that dietary copper deficiency has proinflammatory effects on both neutrophils and the microvascular endothelium that promote neutrophil–endothelial interactions.

Key words: copper, endothelium, inflammation, neutrophils.

Introduction

Dietary copper is known to have an important role in microvascular control mechanisms. These include nitric oxide (NO)-mediated vasodilation[1,2] modulation of mast cell-mediated macromolecular leakage[3] and mechanisms of platelet adhesion to the vascular endothelium.[4] We have recently shown that there is a significant increase in neutrophil accumulation in the lungs of rats fed a copper deficient diet (CuD).[5,6] These results were seen in animals where the copper-deficient diet alone did not affect neutrophil accumulation in several other tissues.[6,7] While the phenomenon demonstrates a role for dietary copper in the interaction between circulating neutrophils and the microvascular endothelium in the lung, the data do not indicate whether the copper-dependent mechanisms involve neutrophil or endothelial cell function. We have hypothesized that dietary copper deficiency primes the lung inflammatory response by priming the circulating neutrophils and the lung microvascular endothelium. Therefore, the aim of this study was to examine neutrophil and endothelial cell mechanisms involved in neutrophil adhesion to and transmigration across the lung endothelium.

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The adhesion process involves a series of events that occur along the vessel wall. Transient interactions between leucocytes and activated endothelium ('rolling') are typically required before the subsequent firm attachment of leucocytes ('sticking') to and transit across the vascular wall.[8] The initial capture and subsequent rolling of leucocytes by the endothelium is primarily mediated by a class of lectin-containing surface adhesion molecules collectively termed 'selectins'. Firm adhesion of leucocytes to the endothelium is primarily mediated by the engagement of leucocyte integrins to members of the immunoglobulin superfamily on the endothelial cell surface.

The neutrophil-endothelial cell adhesion process then activates an intracellular signalling pathway that causes cytoskeletal remodeling within the endothelial cells. This remodeling is required for the neutrophil to migrate along and through the endothelium.[9] It is not clear whether the neutrophil emigration occurs between the endothelial cells (paracellular pathway) or through pores in the endothelial cells (transcellular pathway) but both pathways involve the polymerization of endothelial F-actin.[10]

Materials and Methods

Animals and diet

This project was approved by the University of Louisville Animal Care and Use Committee. Male weanling Sprague-Dawley rats were purchased from Charles River Breeding Laboratories (Wilmington,

MA, USA). On arrival, rats were housed individually in stainless steel cages in a temperature- and humidity-controlled room with a 12 h light-dark cycle. The rats were given free access to distilled water and to one of two purified diets for 4 weeks. The basal diet was a casein-sucrose-cornstarch-based diet (no. TD 84469, Teklad Test Diets, Madison, WI, USA) containing all known essential vitamins and minerals except for copper and iron. The copper-adequate (CuA) diet consisted of the basal diet (940 g/kg of total diet) with safflower oil (50 g/kg) and a copper-iron mineral mix that provided 0.22 g of ferric citrate (16% Fe) and 24 mg of $CuSO_4$ H_2O per kilogram of diet. The copper-deficient diet was the same except for replacement of copper with cornstarch in the mineral mix. Diet analysis by atomic absorption spectrophotometry indicated that the copper-adequate diet contained 6.05 mg copper/kg diet and the copper-deficient diet contained 0.26 mg copper/kg diet. Parallel assays of National Institute of Standards and Technology (NIST; Gaithersburg, MD, USA) reference samples (citrus leaves, no. 1572) yielded values within the specified range, which validated our copper assays.

Hepatic copper determination

The median lobe of the liver was removed, weighed and frozen at –20°C for subsequent copper analysis. Tissues were lyophilized and digested in nitric acid and hydrogen peroxide.[11] Hepatic copper concentrations of individual rats were assessed by using inductively coupled argon plasma emission spectrometry (Jarrell-Ash, model 1140, Waltham, MA, USA). Parallel assays of reference samples (no. 1477a, bovine liver) from the NIST-yielded mineral contents within the specified range.

Neutrophil isolation

Animals (total $n = 25$ CuA and $n = 23$ CuD) were anaesthetized with sodium pentobarbital (50 mg/kg of body weight; i.p.). The blood was withdrawn by venipuncture of the vena cava by using syringes containing sodium citrate anticoagulant (final concentration of 10.9 mmol/L) with a ratio of 1 part anticoagulant to 9 parts blood. A total of 5.5 mL of freshly collected blood and the anticoagulant mixture was carefully layered on the top of the same volume (5.5 mL) of Polymorphprep (Nycomed Pharma, Oslo, Norway) in a 15 mL centrifuge tube. The sample was centrifuged at 550 ×g for 35 min at room temperature (~ 21°C). After the centrifugation, neutrophils were carefully collected from the lower band at the sample/medium interface by using a Pasteur pipette. Red blood cells were lysed by the addition of 0.5 mL of deionized water for 30 s and osmolarity was restored by adding 0.5 mL of 2 times concentrated PBS. The cells were washed twice in (PBS) containing 0.5% BSA.

Endothelial cell culture

Human lung microvascular endothelial cells (HMVEC-L; Clonetics, San Diego, CA, USA) were purchased at the 4th passage. The cells were grown in a complete medium (EGM-2 MV BulletKit, Clonetics) until they reached the 7th passage. Then the cells were isolated and cryopreserved until use. Nuclepore Track-Etch polycarbonate membranes (22 × 40 mm in size, 5 μm pore size and pore density of $2 \times 10^6/cm^2$; Whatman, Clifton, NJ, USA) were coated with fibronectin (Sigma Chemical, St Louis, MO, USA) for 1 h. After the fibronectin treatment, the membranes were seeded with HMVEC-L cells. The cells were grown in EGM-2 MV medium until they formed a complete monolayer. To confirm the cell confluence on the membrane, the cells in a test well were labelled with fluorescence (2′,7′-bis [2-carboxyethyl]-5-[and-6]-carboxyfluorescein, acetoxymethyl ester; [BCECF, AM; Molecular Probes, Eugene, OR, USA]) and observed under a microscope for absence of gaps between the cells.

Protocols

Neutrophil extravasation assay

In the first series of experiments the extravasation of neutrophils from both CuA ($n = 6$) and CuD ($n = 5$) rats through a monolayer of normal lung microvascular endothelial cells was studied by using the method of Arthington *et al*.[12] For this protocol, the neutrophils were fluorescently labelled to enhance their visibility. The cell pellet was resuspended in 1 mL of PBS and 5 μL of BCECF, AM was added. The cell suspension was incubated at 37°C for 30 min with slow agitation. After the incubation, to remove excess fluorescence, neutrophils were centrifuged at 500 ×*g* for 5 min, the supernatant was discarded and the cells were suspended in 1 mL of PBS containing 0.5% BSA. The neutrophils labelled with the fluorescence were counted in a haemocytometer. The haemocytometer was placed on the stage of a Carl Zeiss Axioscope FS upright fluorescent microscope (Carl Zeiss, Jena, Germany) equipped with 40× water immersible, UV transmissible fluorescent objective (numerical aperture 0.75). Fluorescence on the cell surface was visualized by using epi-illumination with blue light (excitation at 495 nm wavelength) from a mercury arc lamp and observation of the fluorescence at 520 nm emission wavelength. We did not find contamination of our samples with fluorescently-labelled erythrocytes or with platelets. For the experimentation, the number of cells was adjusted to 10^6 cells per ml of PBS-0.5% BSA solution.

Phosphate-buffered saline-bovine serum albumin (200 μL) containing *N-FORMYL-MET-LEU-PHE* (*f*MLP; 10^{-7} M) chemoattractant peptide was placed on the bottom glass plate of the flow chamber RC-30 (Warner Instruments, Hamden, CT, USA). The HMVEC-L cell coated polycarbonate membranes were placed on top of the bottom plate of the flow chamber and covered with a silicon gasket (375 μm thickness). The width of the gasket slit was 3 mm. Fluorescently labelled neutrophils were perfused through the chamber by using a Mini-infusion pump (Harvard Apparatus, Holliston, MA, USA). The flow rate was set at 0.4 mL/min so that the wall shear rate in the chamber was about 100 s^{-1}, similar to the shear rate in venules. The flow rate calculations were done according to the method described earlier.[13] Prior to the neutrophil perfusion the lung cells were washed with PBS for 2 min. Then the neutrophil suspension (10^6 cells/mL) was perfused through the chamber for 5 min. At the end of the neutrophil perfusion the membrane was washed with PBS solution for another 5 min. Then the membrane was removed and placed under the microscope (Olympus, with objective 20×) upside-down for the analysis. The number of fluorescently labelled neutrophils that went through the membrane and were present on the membrane surface was counted all along the gasket path. Neutrophil extravasation was presented as number of neutrophils per mm^2 of the membrane surface exposed to the flow.

CD11b expression on neutrophils

Fluorescein isothiocyanate (FITC)-conjugated mouse antirat antibodies against CD11b (clone OX-42) were purchased from Pharmingen (San Diego, CA, USA). Thirty μL of PBS containing 2% fetal bovine serum (FBS) was added to 12 × 75 mm capped conical tubes. Four μl of FITC-conjugated rat antimouse CD11b antibody (0.5 mg/mL) was added to each tube. Tubes for isotype controls (rat antimouse IgG_{2b},κ) were prepared simultaneously. Fifty μl of washed neutrophil suspension (1.0×10^6 cells) was added to each tube and mixed gently. Cells were incubated on ice for 45 min, washed once in cold PBS-2% FBS, and resuspended in 450 μL cold 2% paraformaldehyde solution. All

steps were performed at 4°C. Fixed cells were stored at 4°C until FACS analysis within 3 days.

Fluorescein-isothiocyanate fluorescence was read by a FACScan flow cytometer with argon laser (Becton Dickinson, San Jose, CA, USA). Data was collected for 10 000 cells using LYSIS II software (version 2.0, Becton Dickinson).

Data analysis was performed using WinMDI Flow Cytometry Application (Build no. 1301-19-2000, Version 2.8). Histograms were used to set the boundaries of control samples labelled with FITC isotype antibodies. The number of events in the marked region of specifically bound antibody was expressed as a percentage of the total number of cells analysed (10 000). The mean fluorescence of the population was also recorded as an index of the number of cell adhesion molecules per cell.

Neutrophil adhesion to P-selectin

Following a procedure described elsewhere,[14] microscope glass plates (22 × 40 mm) were immersed in nitric acid (50%, v/v, in distilled water) for 24 h. Then the plates were washed twice in anhydrous acetone. After the washing, they were immersed twice in 3-aminopropyltrie-thoxy-saline (APES; Sigma) for 1 min The plates were rinsed with acetone once and then three times with deionized distilled water. After drying them at 37°C, they were kept in a closed container until use.

One side of the APES coated plates was covered with PBS containing purified P-selectin (10 μg/mL, Calbiochem, La Jolla, CA, USA) and incubated for 1 h at 37°C.[15] The plates were then incubated with PBS-1% BSA solution for another hour at 37°C to block any free protein binding sites.

P-selectin coated plates were placed in the flow chamber RC-30 as bottom plates and covered with a silicon gasket (375 μm thickness) with a slit width of 3 mm. The neutrophil suspension was confined in the container connected to the chamber with inlet and outlet tubing. Fluorescently labelled neutrophils were perfused through the chamber by using a microperfusion pump FCS2 (Bioptech, Butler, PA, USA). The flow rate was set at 0.19 mL/min so that the wall shear rate in the chamber was about 70 s^{-1}, similar to the shear rate values in venules. The flow chamber was placed under the microscope with 20× objective and charge-coupled device (CCD) camera. Movements and adhesion of neutrophils were observed on a TV monitor (Sony, Tokyo, Japan) and videotaped for later analysis.

Prior to perfusion of the neutrophil suspension in PBS-0.1% BSA, the PBS-0.1% BSA solution alone was perfused through the chamber for 5 min. The behaviour of the perfused neutrophils was analysed in 30 s increments during the first 5 min of perfusion through the flow chamber. During the analysis, the total number of clearly visible neutrophils (the cells in the focal plane) were counted in the recorded fields (5–10 fields) and a per field average was determined. The rolling neutrophils were identified as the cells moving with significantly slower speed than the other free-flowing neutrophils in the same focal plane. Although there were relatively slower and faster rolling cells, they were all counted as rolling neutrophils and averaged per field. The neutrophil rolling is presented as the percentile ratio of the rolling neutrophils to the average number of all moving neutrophils in the same focal plane. The number of adhered neutrophils was counted in these fields, averaged and expressed as a percentage of the total number of the flowing neutrophils during the 30 s observation window.

Neutrophil migration under agarose

Four well culture plates were filled with agarose solution containing MEM and 5% FBS (both from Gibco, Rockville, MD, USA) following the modified method after Nelson *et al*.[16] Prior to the experimentation, six series of three wells 3 mm in diameter and spaced 3 mm apart were cut in the agarose layer in each plate. The centre well of each three-well series received a 20 μL aliquot of neutrophil suspension (10^6 cells/mL) in Medium 199 (Gibco). The outer well received 20 μL of either *f*MLP (10^{-9} M) or interleukin-8 (IL-8, 10^{-8}M) both dissolved in Medium 199. These doses were chosen based on the work by Heit *et al*.[17] The inner well received 20 μL of non-chemotactic vehicle Medium 199.

The culture plate was incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air for 1 h. After the incubation the wells were filled with 3 mL of methanol for 30 min and then with formalin for 30 min The agarose was removed, the cells were stained with Giemsa for 5 min, washed with water, and air-dried for neutrophil migration observation under the microscope. The distances from the margin of the middle well to the neutrophil migrating the farthest toward the outer well (directional migration) and to the neutrophil migrating the farthest toward the inner well (random migration) were recorded. Neutrophil migration (μm) was calculated by subtracting the random migration distance from the directional migration distance.

Endothelial cell cytoskeletal F-actin distribution assay

HMVEC-L were grown to confluency in 12 well culture plates. To make cells copper-deficient, the cells in six wells were treated with tetraethylenepentamine (TEPA; 50 μmol/L) containing medium for 72 h.[13] Prior to experimentation, the cells were washed with Hanks balanced salt solution (HBSS) to remove the medium. Three wells in each group (copper-adequate and copper-deficient) were treated with histamine (10^{-6} M) for 15 min at 37°C. Then the well contents were aspirated and the cells were incubated with BODIPY-Phalloidin (10 U, Molecular Probes) and lysopalmitoylphosphatidylcholine (100 μg/mL) dissolved in 3.7% formaldehyde for 30 min at 4°C in the dark.[18] After the incubation the cells were washed three times with HBSS. The digital images of the formed intercellular F-actin were recorded with a Carl Zeiss Axiovert-100 Microscope (with 10× objective) equipped with a rhodamine filter (546 nm excitation and 590 nm emission). The images were compared according to the following criteria: (i) presence of nonactin staining areas; (ii) loss of individual stress fibres; (iii) increased peripheral banding F-actin; and (iv) presence of actin foci.

To detect the copper-deficiency-induced formation of F-actin fibres, total fluorescence intensity was assessed for each well by analysing three random fields by using Matrox Inspector Image Analysis software (Matrox Imaging, Dorval, QC, Canada).

Statistics

All data are expressed as mean ± SE. Data were analysed with a one-way or a two-way analysis of variance with subsequent Student-Newman-Keuls test. Differences were considered significant when $p < 0.05$.

Results

Rats that were fed a diet deficient in copper for 4 weeks developed anaemia and had significantly lower liver copper concentration than the rats fed a copper-adequate diet (Table 1). However, growth rate of the rats was not different as indicated by body weight at the time of experimentation (Table 1).

Polymorphonuclear leucocytes extravasation assay

This assay compared the ability of neutrophils from copper-adequate and copper-deficient rats to transmigrate across a

Table 1 Body weight and copper status indices of rats fed the copper-adequate and copper-deficient diets for 4 weeks

Variable	Copper-adequate ($n = 25$)	Copper-deficient ($n = 23$)
Body wt (g)	233 ± 6	211 ± 5
Liver copper (μg/g dry wt)	13.34 ± 0.43	2.22 ± 0.25*
Hematocrit (%)	45.3 ± 1.0	29.7 ± 1.3*

Values are mean ± SEM. *$P < 0.05$.

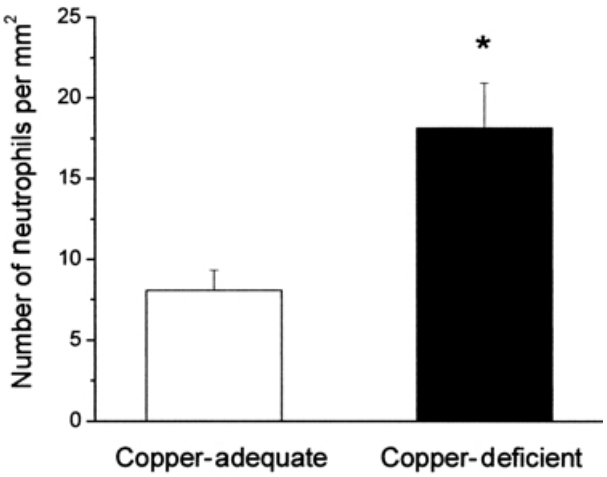

Figure 1 Extravasation of neutrophils from copper-adequate ($n = 6$) and copper-deficient ($n = 5$) rats through a monolayer of cultured lung microvascular endothelial cells in response to *f*MLP (10^{-7} M). Values are mean ± SEM. *$P < 0.05$ for comparison between dietary groups.

monolayer of lung microvascular endothelial cells. In response to the chemoattractant *f*MLP, twice as many copper-deficient neutrophils crossed the endothelial layer compared to the copper-adequate group (Fig. 1). Because there was only a difference in the neutrophil copper status and not in the endothelial cell copper status, the results suggest that dietary copper deficiency promotes transendothelial migration of neutrophils.

CD11b expression on neutrophils

Neutrophils were isolated from copper-adequate and copper-deficient rats and assayed for the expression of the adhesion molecule CD11b. The purity of the neutrophil isolation is demonstrated by the high number of cells expressing the ligand (95.8% of the adequate group and 86.3% of the deficient group). FACS analysis showed that the mean fluorescent intensity per 10 000 cell sample was eight times greater in the copper-deficient group compared to the copper-adequate controls (Fig. 2). These results demonstrate a diet-induced increase in the expression of the neutrophil adhesion molecule that invokes firm adhesion and extravasation.

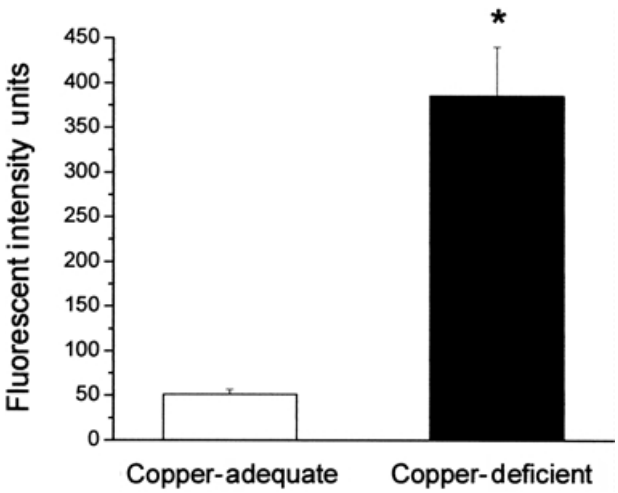

Figure 2 Neutrophil expression of CD11b as determined with FITC-conjugated anti-CD11b mAb. Neutrophils were from copper-adequate ($n = 8$) and copper-deficient ($n = 7$) rats. Values are mean ± SEM. *$P < 0.05$ for comparison between dietary groups.

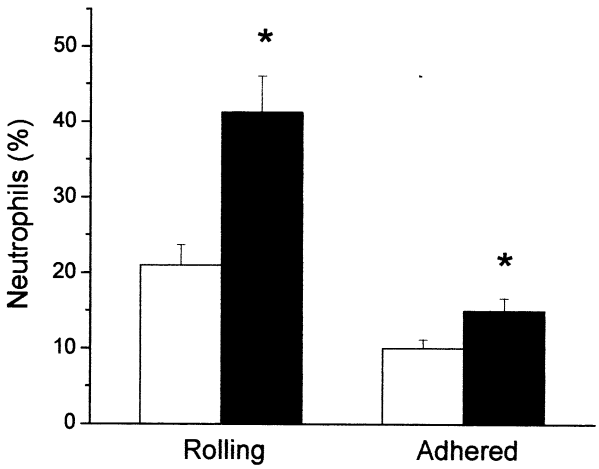

Figure 3 Determination of transient and firm adhesion of neutrophils to P-selectin under flow conditions. Neutrophils were from copper-adequate ($n = 7$) and copper-deficient ($n = 7$) rats. Comparison between groups was by one-way analysis of variance (ANOVA). Values are mean ± SEM. *$P < 0.05$ for comparison between dietary groups. □, copper-adequate; ■, copper-deficient.

Neutrophil adhesion to P-selectin

This protocol focused on the neutrophil component of the adhesion process by removing the interaction with endothelial cells and using P-selectin instead. The adhesion of neutrophils isolated from copper-deficient rats was significantly greater than the copper-adequate control group. This is seen in both the transient (rolling) and firm adhesion to the P-selectin (Fig. 3). These results suggest that dietary copper deficiency promotes neutrophil adhesion by activation of the circulating neutrophils.

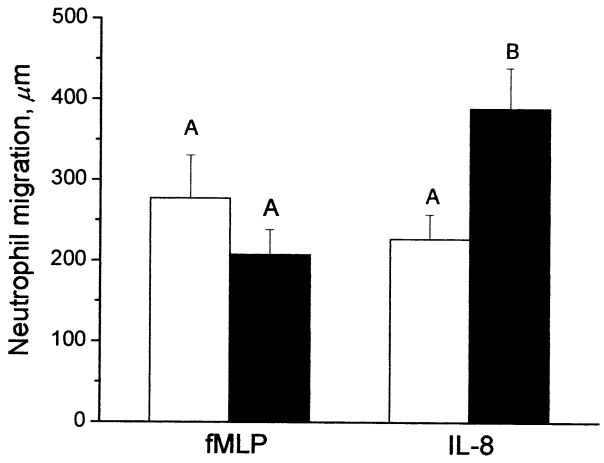

Figure 4 Neutrophil migration under agarose. Neutrophils were from copper-adequate ($n = 4$) and copper-deficient ($n = 4$) rats chemotaxis was to 10^{-9} M *f*MLP and 10^{-8} M IL-8. Values are mean ± SEM. Bars with different letters are significantly different ($P < 0.05$). □, copper-adequate; ■, copper-deficient.

Neutrophil migration under agarose

Cellular migration in response to the chemotactic agents *f*MLP and IL-8 were compared between copper-adequate and copper-deficient neutrophils. The results show that there is no difference in the chemotactic response to CD11b/CD18-dependent *f*MLP between dietary groups (Fig. 4). However, in response to IL-8, which is CD11b/CD18-independent, the neutrophil migration was significantly greater in the copper-deficient group compared to the copper-adequate group (Fig. 4). These results demonstrate that dietary copper deficiency enhances neutrophil chemotaxis and that the increased migration is chemotaxin-specific.

Endothelial cell F-actin distribution assay

Alterations in endothelial F-actin were monitored by the binding of BODIPY-phalloidin to actin in normal and copper-chelated lung microvascular endothelial cells. Figure 5 is a representative fluorescence photomicrograph showing the effects of either control (HBSS) or 10^{-6} M histamine on F-actin distribution. Analysis of the fluorescent intensity showed that there was significantly greater F-actin staining in the copper-chelated endothelial cells than in the copper-adequate group under the unstimulated control conditions (Fig. 6). The addition of histamine to the cells significantly increased the F-actin staining in both groups with the copper-chelated group again having more staining than the copper-adequate group (Fig. 6). Comparison between the copper-chelated control group and the copper-adequate and histamine group suggests that copper deficiency promotes F-actin polymerization similar to histamine (Fig. 6). The copper-chelated and histamine group also shows evidence of gaps between adjacent cells (Fig. 5D), possibly the result of endothelial cell contraction. These results suggest that inadequate copper content in endothelial cells primes the contractile mechanism associated with F-actin polymerization. The result of reduced copper is similar to that seen in the response to histamine in normal cultured endothelial cells.

Discussion

Neutrophil sequestration and emigration are important features of the inflammatory response in the microcirculation. The processes involve both neutrophil and endothelial cell mechanisms and the interactions between the two cell types. In the current study, the overall adhesion, migration and emigration of neutrophils through a monolayer of lung microvascular endothelial cells was seen to be greater for copper-deficient neutrophils than for the copper-adequate controls (Fig. 1). The results from this flow chamber study are similar to results previously reported in the *in vivo* lung microvasculature of copper-adequate and copper-deficient rats.[5,6] We have also documented an increased extravasation of copper-deficient neutrophils through a monolayer of coronary microvascular endothelial cells under static conditions.[6] Combined, these data demonstrate a role for dietary copper in the modulation of neutrophil–endothelial cell interactions but do not indicate which mechanisms are copper-dependent.

In the first set of experiments designed to independently examine the neutrophil and endothelial cell mechanisms, the data demonstrate that the leucocyte adhesion molecule CD11b is significantly increased on the surface of neutrophils from copper-deficient rats (Fig. 2). The increased presence of the CD11b, which is part of the CD11/CD18 adhesion complex, is important in both the firm adhesion of the neutrophil to the endothelial cell[19] and in emigration through the endothelium in the rat lung.[20] While the mechanism of the augmented CD11b expression is not known, it is clear that dietary copper deficiency has a proinflammatory effect on neutrophil adhesion and emigration by increasing the presence of the adhesion molecule.

The effect of copper deficiency on neutrophil adhesion can be seen in the next experiment where the transient and firm adhesion of copper-adequate and copper-deficient neutrophils were studied. Under flow conditions similar to that seen in venules, the results demonstrate that neutrophils from copper-deficient rats 'roll' and 'stick' significantly more than those from copper-adequate rats (Fig. 3). Because these experiments were done with P-selectin instead of endothelial cells, the results can be attributed to the role of copper in neutrophil function. The results suggest that copper deficiency has a stimulatory effect on the adhesive properties of neutrophils. These results further support the premise that dietary restriction of copper has a proinflammatory effect on neutrophil–endothelial interactions with neutrophil-specific mechanisms.

While the current *in vitro* data demonstrates that copper restriction causes changes in the adhesive properties of neutrophils, we have shown that the accumulation of neutrophils *in vivo* is also dependent on the tissue type.[6] For example, we have observed that dietary copper deficiency does not significantly change the number of rolling and sticking neutrophils[7] or the accumulation of neutrophils in postcapillary venules of the rat cremaster muscle.[6] This is in contrast to the significantly greater accumulation of neutrophils in the lungs of the same animals.[6] These differences in neutrophil adhesion between the lung and the cremaster muscle probably relate to differences in signalling pathways and adhesion mechanisms in the lung compared to the systemic microcirculation.[21]

Once neutrophils have adhered to endothelial cells, they then flatten and migrate toward the junctions. This biomechanical movement was tested in neutrophils from copper-adequate

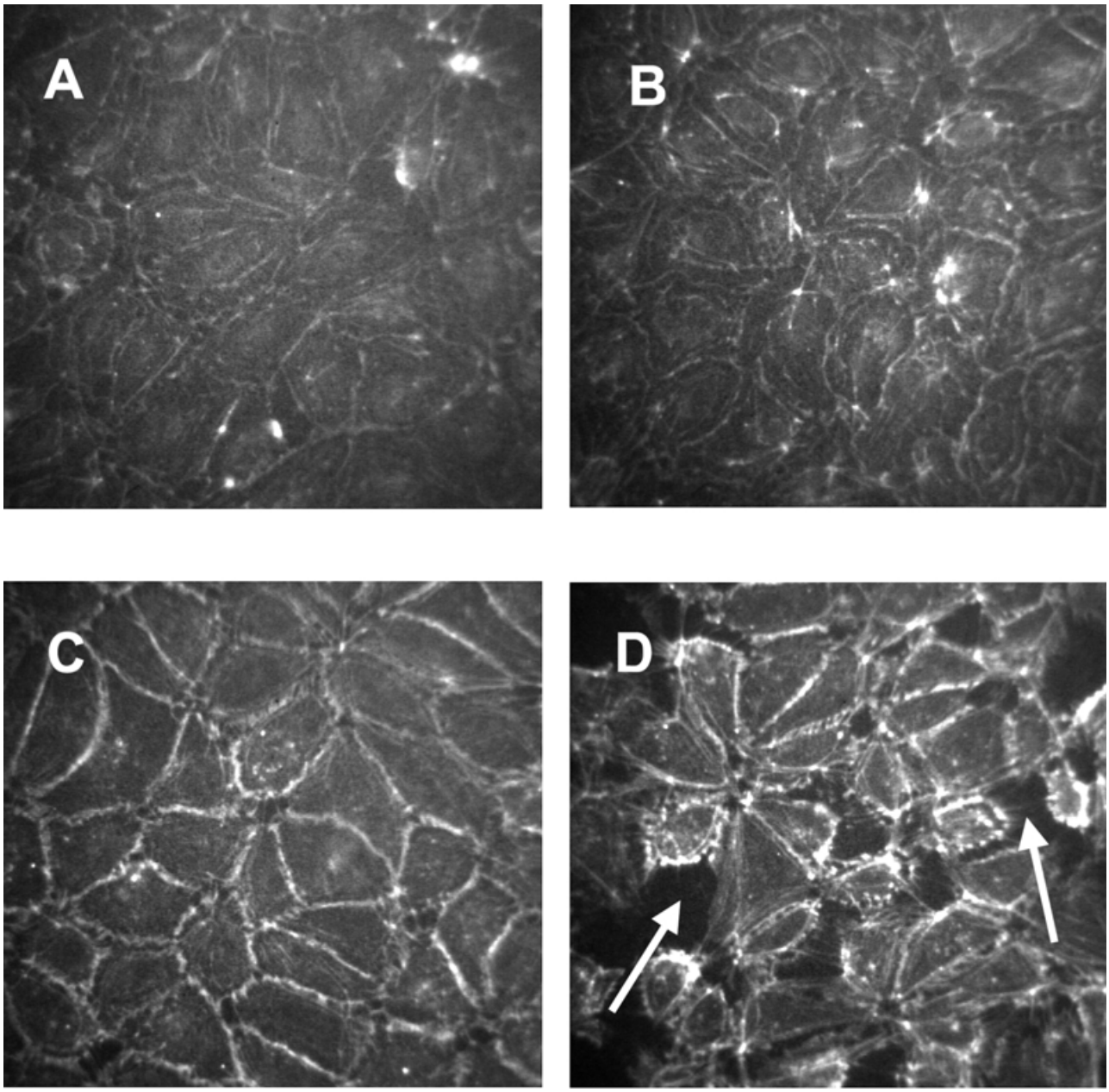

Figure 5 The distribution of F-actin in cultured lung microvascular endothelial cells. Cells were copper-adequate (A), copper-chelated (B), copper-adequate stimulated with 10^{-6} M histamine (C) and copper-chelated with histamine (D). Endothelial gaps are indicated with arrows.

and copper-deficient rats independent of the presence of endothelial cells. The results showed that there is no difference in migration to *f*MLP between the dietary groups (Fig. 4) but that the copper-deficient neutrophils migrated farther in response to IL-8 (Fig. 4). This represents another change in the function of copper-deficient neutrophils that may exaggerate an inflammatory response.

Migration of neutrophils to the two chemoattractants used represent two different signalling pathways. Neutrophil migration through pulmonary arterial endothelial cells in response to *f*MLP is CD11/CD18-dependent while IL-8-induced migration is CD11/CD18-independent.[9] The current data suggests that although CD11b expression is greater in the copper-deficient group (Fig. 2), the chemotactic response of copper-deficient neutrophils is more sensitive to IL-8. We have previously proposed that the CD11/CD18-independent pathway may be enhanced in the copper-deficient rat lung.[6] Our conclusion was based on data showing that there was greater neutrophil accumulation in the copper-deficient lung without an increase in the expression of intercellular adhesion molecule-1 (ICAM-1), which is a major endothelial cell ligand for the CD11/CD18 complex. The current data adds further evidence for exaggerated CD11/CD18-independent signalling during copper deficiency.

Neutrophil-endothelial adhesion also results in functional changes in the pulmonary endothelial cells. Ligation of ICAM-1 by neutrophils results in cytoskeletal remodeling in the endothelial cells required for neutrophils to migrate to the

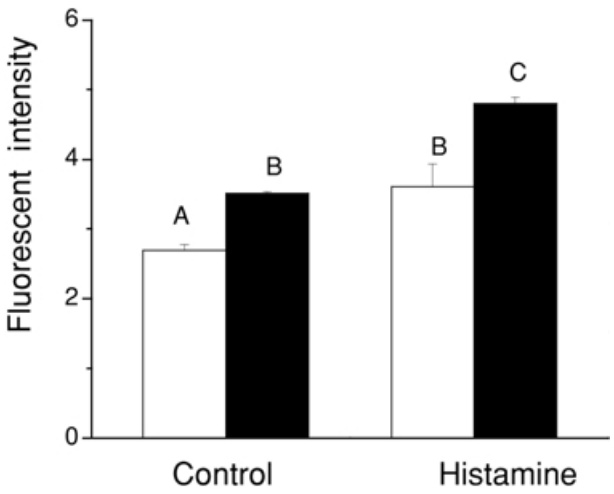

Figure 6 Comparison of the fluorescent intensity of BODIPY-Phalloidin stained F-actin in cultured lung microvascular endothelial cells. The cells were unstimulated (control) or stimulated with 10^{-6} M histamine. Two-way ANOVA indicated significant main effects of diet and histamine and interaction between diet and histamine. Bars with different letters are significantly different ($P < 0.05$, Student-Newman-Keuls method). Values are mean ± SEM. Body weight and copper status indices of rats fed the copper-adequate and copper-deficient diets for 4 weeks. □, copper-adequate; ■, copper-deficient.

borders of the endothelial cells.[9] The cytoskeletal remodeling consists of polymerization of F-actin causing changes in endothelial cell shape and surface characteristics.[10] These cytoskelatal changes may alter the paracellular junctions or widen transcellular pores[23] allowing neutrophils to leave the vascular space and emigrate into the interstitium.

In the current study, copper-chelated pulmonary microvascular endothelial cells had significantly greater fluorescently labelled F-actin compared to the control cells (Fig. 6). The addition of histamine as an inflammatory mediator significantly increased the amount of F-actin in both groups with the greatest amount being in the copper-chelated group (Fig. 6). Interestingly, the amount of F-actin polymerization caused by the removal of copper equaled the copper-adequate group stimulated with histamine. While no changes in endothelial barrier integrity have been seen in the *in vivo* microvasculature of copper deficient rats[5,24] the current results suggest a priming of the endothelial cell contractile machinery.

Increased F-actin has been seen in copper-deficient platelets[25] as well as the endothelial cells (Figs 5 and 6) but the mechanism of the increased F-actin in the copper-chelated cells is not known. Superoxide has been linked to the F-actin polymerization and cytoskeletal remodeling seen in neutrophil–endothelial interactions.[9] Chelation of copper from cultured microvascular endothelial cells not only depletes the cellular copper concentration but also significantly decreases the activity of copper-zinc superoxide dismutase (Cu,Zn-SOD).[13] Therefore, we speculate that a build-up of superoxide anion because of the lost Cu,Zn-SOD activity promotes F-actin polymerization in the copper-deficient endothelium.

In summary, we have shown that dietary copper-deficiency alters normal neutrophil–endothelial interactions by affecting both the neutrophils and endothelial components of the interactions. The neutrophil adhesion is greater, possibly because of the greater expression of the CD11b adhesion molecule, copper-deficient neutrophil migration is increased by greater sensitivity to a CD11/CD18-independent signalling pathway, and emigration through endothelial cells is likely promoted by the altered F-actin cytoskeleton. The effects of these changes can be seen *in vivo* in the lungs of copper deficient rats where there is a significantly greater accumulation of neutrophils caused by a dietary copper-restriction.[5,6] The results suggest a proinflammatory effect of copper deficiency on mechanisms of neutrophil sequestration and emigration.

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